Crystallization for the isolation and purification of artemisinin from crude extracts of Artemisia annua: feasibiity and challenges

Qu, Haiyan; Christensen, Kathrine Bisgaard; Fretté, Xavier; Tian, Fang; Grevsen, Kai; Rantanen, Jukka Tapio; Christensen, Lars Porskjær

Published in:
BIWIC 2009, Proceeding of the 16th International Workshop on Industrial Crystallization

Publication date:
2009

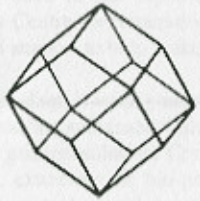
Document Version
Final published version

BIWIC 2009

Proceeding of the 16th International Workshop

on

Industrial Crystallization



September 9-11, 2009

Lappeenranta University of Technology

Lappeenranta, Finland

Edited by

Marjatta Louhi-Kultanen and Henry Hatakka

Lappeenranta University of Technology, Finland

Lappeenranta 2009

BIWIC 2009/16th International Workshop on Industrial Crystallization.
September 9-11, 2009, Department of Chemical Technology,
Lappeenranta University of Technology.
Eds.: Marjatta Louhi-Kultanen, Henry Hatakka
Department of Chemical Technology
Lappeenranta University of Technology
P.O. Box 20 (Skinnarilankatu 34)
FIN-53851 Lappeenranta
Finland
www.lut.fi
ISBN 978-952-214-806-3

ISBN 978-952-214-806-3

Lappeenranta University of Technology, Finland
Digipaino

Preface

It is our great pleasure to host BIWIC 2009, the 16th International Workshop on Industrial Crystallization, at Lappeenranta University of Technology. The event continues the successful series of meetings initiated primarily by Joachim Ulrich and follows on from gatherings held in Bremen, Delft, Rouen, Gyeongju, Halle, Cape Town, and last year in Magdeburg. This year BIWIC 2009 brings together over 60 participants from Europe, China, Iran, Japan, Korea and Thailand.

Organization of BIWIC 2009 has been in the capable hands of the scientific and local organization committees and LUT's Centre for Separation Technology, CST. We thank them for their guidance, collaboration and support to help make BIWIC 2009 a success.

Crystallization, the theme of the workshop, is widely used as a purification, concentration and separation method when a pure crystalline substance with specific particle properties needs to be isolated from a multi-component process solution. Crystallization of organic compounds is generally followed by a synthesis, extraction or bio-process. The main challenges facing industrial crystallization are how to control crystal morphology, crystal size distribution and polymorphism.

BIWIC 2009 covers a wide range of interesting and exciting topics. A significant number of BIWIC 2009 contributions are on crystallization of pharmaceutical compounds. An example is the isolation and purification of a drug molecule by crystallization from a traditional Chinese plant. Novel crystallization applications can be found in biotechnology processes, the significance of which can be expected to increase in the near future. It seems that there will be a need to develop new methods to identify and separate potential fine chemicals in bio-refineries for the pharmaceutical, food and chemical industry, or to isolate harmful substances from fermentation broths. A further new application example is the purification of ionic liquids by crystallization. In the food industry, ice crystallization and other melt crystallization methods are used as concentration or purification methods. Spectroscopic analysis methods allow in-line real-time process monitoring and process control. Precipitation of nano-sized crystals is a further topic of BIWIC 2009 presentations. In addition to crystallization, effective down-stream processing (filtration of crystals from mother liquor, drying of crystalline product) is also crucial for the whole isolation process. Other topics of BIWIC 2009 contributions include crystallization of inorganic compounds, crystallization kinetics research, and modelling of crystallization.

We hope this meeting of experts in the field will help crystallize new ideas and approaches. We wish you a fruitful and memorable experience at BIWIC 2009.

Marjatta Louhi-Kultanen and Henry Hatakka

The disadvantage was a polymorphic mixture due to the violent release of the supersaturation. However, the yield was quite high which implies that crystallization from the dispersed phase was complete.

Organic Compound B was crystallized in liquid-liquid system by evaporation in order to produce crystals of micronized grade, and simultaneously for the removal of the main impurity B-V-B. Likewise, supersaturation was achieved by evaporation of more volatile component from the drop and also by cooling the dispersion. As a result, crystal size was 50 % ≤ 13 μm, and the main impurity B-V-B reduced to 1/14 part of the crude material impurity level. According to lab-scale filtration no major problems were expected in scale-up possibly due to high crystallinity of the product. The yield met expectations because of high impurity removal level in the crude Compound B.

4 Conclusion

The formation polymorphic mixture was a problem in case of Compound A crystallization, and spherical agglomeration (SA) or true emulsion solvent diffusion (ESD) method would be more suitable. However , the presented method could be applicable for other compounds.

The presented method can be applied in industrial scale for the case of Compound B crystallization. However, optimization for the removal of impurity B-V-C should be done, e.g. by adding acetone washing or for using optimized portion of acetone in dispersed phase.

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Crystallization for the isolation and purification of artemisinin from crude extracts of Artemisia annua: feasibility and challenges

H. Qu[1], K. B. Christensen[1], X. C. Fretté[1], F. Tian[2], K. Grevsen[3], J. Rantanen[2], L. P. Christensen[1]
[1] Institute of Chemical Engineering, Biotechnology and Environmental Technology, University of Southern Denmark, Denmark
[2]Department of Pharmaceutics and Analytical Chemistry, Faculty of Pharmaceutical Sciences, University of Copenhagen, Denmark
[3]Department of Horticulture, Faculty of Agricultural Sciences, University of Aarhus, Aarslev, Denmark
haq@kbm.sdu.dk

In this work, the feasibility of using a chromatography–crystallization hybrid separation process for the isolation and purification of artemisinin from the herbal plant Artemisia annua *was studied. A two-step anti-solvent crystallization process was proposed based on the solubility of artemisinin in different solvents. The first step crystallization process was to remove the impurities from the solution and the second step to isolate artemisinin. The crystallization behaviour of the two polymorphs of artemisinin during the anti-solvent crystallization process was also studied by performing crystallization in solutions of artemisinin in acetonitrile and acetone, respectively. It was observed that the formation of the two polymorphs of artemisinin in acetone solution follows the Ostwald's rule of stages. A fast feeding of the anti-solvent (water) resulted in the crystallization of the metastable triclinic form while the stable orthorhombic form crystallized out when water was fed slowly. Finally, a chromatography–crystallization hybrid separation process was proposed and tested. The results of the present work demonstrated a great potential of combining the advantages of column chromatography and crystallization to improve the efficiency of the isolation of artemisinin from the plant* Artemisia annua.

1 Introduction

Artemisinin is a sesquiterpene lactone peroxide derived from the Chinese medicinal herb *Artemisia annua*. It is the most effective drug against chloroquine-resistant *Plasmodium falciparum* infection and cerebral malaria [1–3]. In addition, the anticancer activity of artemisinin and its activity against other parasite species have also been reported [4]. Consequently, the worldwide demand of artemisinin is continuously increasing. The total synthesis of artemisinin has been discovered in the 1980s [5], however, the complex steps and the relatively low yield of the synthetic approach has limited its application to the real industrial production of artemisinin. Currently, the extraction from *A. annua* is the only source of artemisinin in the market. In order to develop a reliable market supply of artemisinin with a reasonable price, it is paramount importance to do the research towards the following two directions: firstly, to increase the concentration of artemisinin in *A. annua* by optimizing the cultivation and harvest conditions, selecting high yielding cultivars, or creating transgenic plants; secondly, to improve the efficiency of the production process of artemisinin from the plant materials. Several research programs have been set up aiming at cultivating *A. annua* containing more artemisinin. However, little has been done on the optimization of the isolation process to improve artemisinin recovery yields from plant extracts.

A typical isolation and purification process for artemisinin from *A. annua* consists of the extraction of the plant material and the separation of artemisinin from other compounds extracted from the plant. The separation of artemisinin from the extracts is very challenging due to the extreme complexity of the extracts and the presence of compounds in the extracts of which many have chemical structures very similar to artemisinin (as shown in Fig. 1) and hence similar chemical and chromatographic characteristics [6]. Purification of the crude extract by chromatographic methods followed by crystallization of the pure artemisinin is usually involved in separated steps. In principle, complete separation of the compounds from the crude extracts can be achieved by column chromatography (CC) using a sufficiently long column. However, the capital and operational costs of the separation process can be very high in such cases, and thus increase the total manufacturing cost of the product. In practice many of the extraction plants located in the traditional cultivation and processing areas of *A. annua*, e.g., China and Vietnam have excluded the chromatographic purification step and solely use crystallization for separation. In order to produce a high-purity product, artemisinin has to be re-crystallized several times, which leads to significant loss of the product. It has been reported from two extraction plants in Vietnam that the yield of artemisinin was approximately 0.2–0.3 % based on dry plant. However, the analysis of these *A. annua* plants showed that the concentration of artemisinin in the dry plant was 0.8–1 % [7]. Obviously, the profitability of this process might become unacceptable in a country where the cost for plant cultivation is much higher than in Vietnam and the environmental regulations are more strict. The development of an artemisinin production process in such countries requires a well-designed and more efficient isolation and purification procedure.

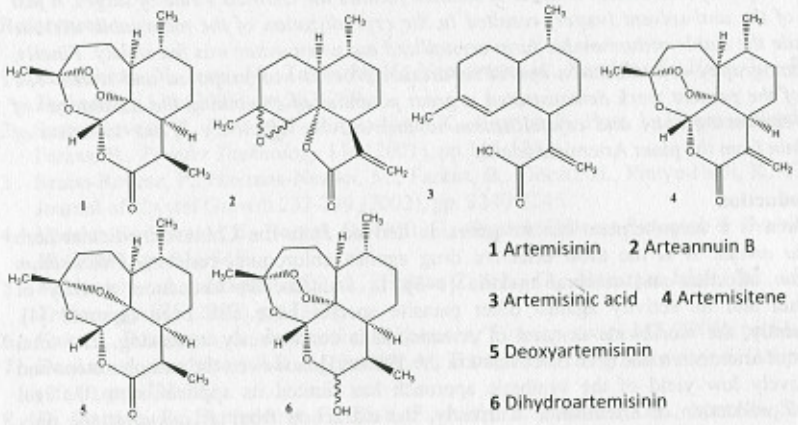

Fig. 1 Chemical structures of artemisinin and related compounds that can be extracted from *A. annua*.

As powerful separation techniques, both chromatography and crystallization have their own advantages and limitations. Chromatography is capable of fractionating an extreme complex mixture containing hundreds of compounds, however, the complete separation of the compounds requires long column and thus high capital and operational costs. On the other hand, crystallization is appropriate for the isolation of a product with high purity from a multi-components mixture, but the separation effect and yield are strongly affected by the presence

of other impurity compounds, especially those possessing similar molecular structures as the crystallizing product. Therefore, the hybrid between chromatography and crystallization can have a synergistic effect, which can be developed as a robust and efficient separation approach for the isolation of bioactive compounds from a complex multi-components mixture [8].

In addition to separation and purification, crystallization is also a technique for solid form selection. Since the different solid forms may have distinct physical and chemical properties, such as solubility, dissolution rate, density, thermal and chemical stability, the control of the polymorphism of the crystalline product during the processing is one of the most important issues in pharmaceutical industry. It has been reported that artemisinin is capable of forming two polymorphs, an orthorhombic form and a triclinic form [9]. However, the crystallization behaviour of the two polymorphs of artemisinin hasn't been studied.

The objective of the present work is to explore the feasibility of combining the advantages of both chromatography and crystallization to generate a hybrid separation process for the isolation and purification of artemisinin from *A. annua*. The solubility of artemisinin in different solvents and solvents mixtures was measured and was then used to design a two-step anti-solvent crystallization procedure. The crystallization behaviour of the two polymorphs of artemisinin during the anti-solvent crystallization process was studied. Based on the solubility and crystallization behaviour of artemisinin, a chromatography–crystallization hybrid separation process was proposed. The feasibility of this hybrid separation process was investigated through a laboratory-scale isolation of artemisinin from an extract of *A. annua*.

2 Materials and methods

2.1 Chemicals

The pure artemisinin was obtained from My Dinh Extraction plant in Vietnam and Xiang Xi Holley Pharmaceutical Co. Ltd. in China. The purity of the material is > 99%. The organic solvents used in all experiments are HPLC grade from Fisher Scientific (Slangerup, Denmark). Water was purified using a SG Ultra Clear Basic UV system (Holm & Halby, Germany).

2.2 Solubility measurement

The solubility of pure artemisinin in various organic solvents and solvent mixtures was measured at room temperature. The organic solvents used include ethanol, methanol, dichloromethane, acetonitrile, acetone, ethyl acetate, hexane, and chloroform. The mixed solvents are binary mixtures of acetonitrile–water and ethanol–water. Ten mL solvent with excess artemisinin was added to a 25 mL flask. The liquid–solid suspension was then kept under mixing in a water bath at 24.5 °C for 2 h. The clear solution was then removed with a syringe filter. One mL clear solution was sampled and after dilution, it was analyzed with an Agilent 1100 series High-Performance Liquid Chromatography (HPLC) equipped with a PDA detector to determine the solubility of artemisinin (conditions: Gemini 3μ C18 110A column, 10 cm×3 mm i.d.; mobile phase, 0.5 mL/min water-acetonitrile (4:6); UV detector 210 nm). The solubility was also determined with a gravimetric method by evaporating the remaining of the clear solution.

2.3 Anti-solvent crystallization of artemisinin

The anti-solvent crystallization of artemisinin was performed by feeding water to the solution of artemisinin in acetone and acetonitrile, respectively. One mL artemisinin–acetone or artemisinin–acetonitrile solution saturated at room temperature was prepared in an 8 mL flask. A magnetic mixer was used to provide the mixing of the solution. Water was fed to the solution with two different feeding modes: in the slow feeding mode, water was fed with a 20-μL pipette at a flow rate of 20 μL/min. The solution became turbid after 30–40 μL water was added. After that, the feeding flow rate of water was increased gradually, and finally 6 mL water was fed to the solution within 1 h. For the fast feeding mode, the 6 mL water was added rapidly with a pipette. The suspension was then filtered and the crystals were dried at room temperature overnight. The X-ray powder diffraction patterns of the crystals were collected with a PANalytical X'Pert PRO X-Ray Diffractometer (Almelo, Netherlands) to identify the polymorphism of the produced crystals.

2.4 Plant material extraction

Artemisia annua was cultivated and harvested at Department of Horticulture, Research Centre Aarslev, University of Aarhus in 2006 and 2007, and was stored at –20 °C until extraction. The plant material was cut into small pieces and extracted with dichloromethane at room temperature for 24 h. The ratio of the plant material and the extraction solvent was 150 g plant/800 mL solvent. The plant material was re-extracted once using similar conditions. The extracts were then combined and dried under vacuum.

2.5 Chromatography–crystallization hybrid separation process

The combined extracts were separated using flash CC on silica gel (silica gel 60, particle size ≤ 0.063 mm) and eluted with dichloromethane. The ratio of solute to absorbent was 1:30. The fractions were monitored by thin layer chromatography (TLC). TLC plates were normal-phase silica gel 60 F254nm 20×20 cm from Merck (KGaA, Germany) and the mobile phase was dichloromethane. Plates were inspected by UV light followed by visualization with Vanillin (30 g vanillin, 500 ml ethanol, 5 ml conc. H_2SO_4). The fractions rich in artemisinin and containing the same compounds were combined and brought to the crystallization step.

3 Results and discussion

Table 1 Solubility of artemisinin in different solvents at room temperature.

Solvent	Gravimetric mehtod (mg/mL)	HPLC method (mg/mL) (n=2)
Dichloromethane	1086.9	974.9±0.3
Acetonitrile	267.7	268.8±0.8
Acetone	242.2	229.3±0.2
Ethanol	31.6	35.1±1.2
Methanol	46.8	40.5±0.1
Ethyl acetate	135.2	121.2±2.6
Chloroform	1061.8	972.9±2.6
Hexane	1.8	-

3.1 Solubility of artemisinin

The solubility of artemisinin in different organic solvents is shown in Table 1 and in Fig. 2 against the Hildebrand solubility parameter of the solvent. Artemisinin has been reported to

be insoluble in water and oil but soluble in most aprotic organic solvents. It can be seen from Fig. 2 that artemisinin is more soluble in the solvents with medium polarity (solubility parameter between 18–24). In addition, the chemical structure of the solvent also significantly affects the solubilization potential of the solvent. Ethyl acetate and acetone have similar solubility parameter as chloroform and dichloromethane, however, the solubility of artemisinin in chloroform and dichloromethane is about 5 times higher than that in ethyl acetate and acetone.

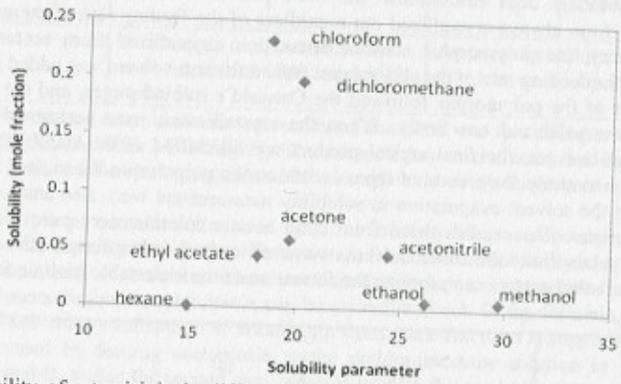

Fig. 2 Solubility of artemisinin in different organic solvents at room temperature (24.5°C) (measured with gravimetric method).

The solubility of artemisinin in aqueous acetonitrile and ethanol solutions were also measured. The results are shown in Fig. 3. The solubility of artemisinin remarkably decreased with increasing water concentration, which suggested the feasibility of anti-solvent crystallization by using water as the anti-solvent.

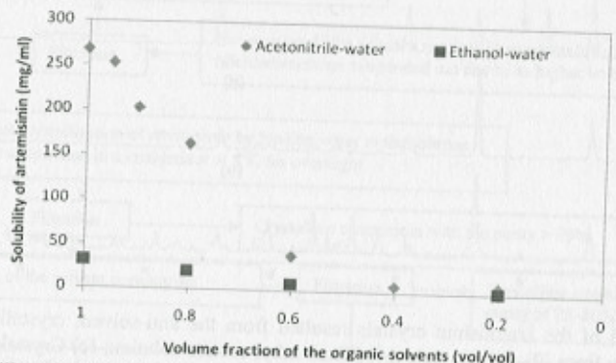

Fig. 3 Solubility of artemisinin in mixed solvents.

3.2 Crystallization behaviour of artemisinin

Artemisinin is capable of forming two different polymorphs, the orthorhombic form with higher density and lower solubility in water is considered as the thermodynamically stable form at room temperature, and the triclinic form is the metastable one. The X-ray Powder Diffraction (XRPD) patterns of the artemisinin crystals resulted from the anti-solvent crystallization from acetone and acetonitrile solutions is shown in Fig. 4. It was observed that the formation of the polymorphs of artemisinin dependent on the solvent and the generation rate of supersaturation. The crystallization of the two polymorphs of artemisinin from acetonitrile solution does not follow the well known Ostwald's rule of stages, the orthorhombic form always crystallized out regardless of the feeding rate of the anti-solvent. On the contrary, the polymorphic state of artemisinin crystallized from acetone solution depended on the feeding rate of the anti-solvent. When the anti-solvent was added rapidly, the crystallization of the polymorphs followed the Ostwald's rule of stages, and the metastable triclinic form crystallized out firstly. When the crystallization was performed with slow feeding of anti-solvent, the final crystal product was identified as the stable orthorhombic form. In order to study the effects of the solvents on the polymorphs formation, the crystals resulted from the solvent evaporation in solubility measurement were also analyzed with X-ray diffractometer. The crystals dried from ethyl acetate solution were pure triclinic form, while the crystals from the other solvents were all orthorhombic form. This observation suggested that ethyl acetate can promote the formation of the metastable triclinic form.

Fig. 4 XRPD of the artemisinin crystals resulted from the anti-solvent crystallizations (a) Triclinic form from [9]; (b) Crystals dried from ethyl acetate solution; (c) Crystals from anti-solvent crystallization in acetone solution with fast anti-solvent feeding; (d) Crystals from anti-solvent crystallization in acetonitrile solution with fast anti-solvent feeding; (e) Orthohombic form from [9].

3.3 Crystallization of artemisinin from column chromatography fractions

The fractions obtained by CC of an extract of *A. annua* and containing artemisinin were classified into four groups and were further combined into four mixtures with the following volume and artemisinin concentrations:

Combined fractions	M1	M2	M3	M4
Volume (ml)	460	580	200	390
Artemisinin concentration (mg/ml)	1.337	0.209	0.113	0.286
Composition	Artemisinin and impurities A and B	Artemisinin and impurity B	Artemisinin	Artemsinin and impurity C

(The concentration of artemisinin in the combined fractions was quantified with a Thermo Scientific LTQXL AP-ESI LC-MS [10]; The composition of the combined fractions was assessed by TLC, and the elution sequence of the compounds are: A-B-Artemisinin-C.)

All of the solutions contain artemisinin and one or two other compounds, which are considered as the impurity in the crystallization step. Based on the solubility of artemisinin in dichloromethane and acetonitrile, when acetonitrile is fed to a saturated artemisinin-dichloromethane solution, no artemisinin can be crystallized out. In the following (Fig. 5), a two-step anti-solvent crystallization of artemisinin from crude fractions is suggested: The first step is performed by feeding acetonitrile to the dichloromethane solution to remove the impurities A and B, and in the second step, water is used as the anti-solvent to crystallize out the artemisinin product.

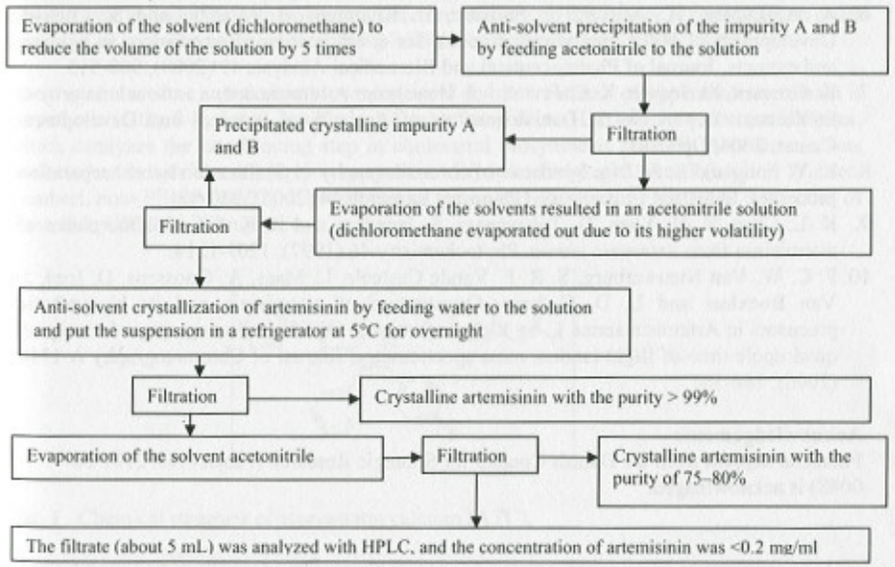

Fig. 5 Schematic process flow diagram of the two-step crystallization process for artemisinin isolation.

Fractions M3 and M4 from the CC fractionation step were not brought to the crystallization step, due to the low total amount of artemisinin in the solutions. The separation of artemisinin in these mixtures will be studied in the future when more *A. annua* extract are fractionated by CC.

4 Conclusions

The traditional herbal medicines could serve as a source of inspiration for drug development, and it has drawn a renewed interest from the pharmaceutical industry recently. The development of drugs from natural plants usually requires the isolation and purification of the target compound from a complex multi-component mixture. The chromatography–crystallization hybrid separation process suggested in the present work might be one of the most promising techniques for this kind of natural product separations.

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Acknowledgements

Financial support from the Danish Council for Strategic Research (Project No. 2101-08-0048) is acknowledged.

New Crystalline Solvates of Atorvastatin Calcium

Yong Suk Jin, Joachim Ulrich

Martin-Luther-Universität Halle-Wittenberg, Zentrum für Ingenieurwissenschaften, Verfahrenstechnik/TVT, D-06099 Halle (Saale), Germany
joachim.ulrich@iw.uni-halle.de, yong.jin@iw.uni-halle.de

Atorvastatin calcium (ATC) is widely prescribed as cholesterol-lowering agent and is the world's best-selling medicine. A large number of crystalline forms of ATC have been published in patents. In this work, two new crystalline solvates were discovered. Common crystallization methods were used to find these forms from amorphous ATC. For characterization the following methods were applied: XRPD, DSC, TG, optical microscopy and FT-Raman spectroscopy. The analytical results were well distinguishable from those of the previously reported crystalline polymorphs, solvates and amorphous forms.

1 Introduction

The phenomenon of polymorphism is important in the pharmaceutical field because different solid forms may exhibit different physical properties related to solubility, stability, dissolution rate, bioavailability and so on [Hal69, Bri99]. Most pharmaceutical products are used in solid forms, thus the investigation of various solid forms is crucial for the selection of the best form for the intended use and improvement of the performance of drugs [Hil06]. In addition, the transformation behavior of polymorphs and solvates in crystallization are often complicated and difficult to understand completely. Therefore, a study on this matter is significant in order to design or control a process to get the solid form with desired properties [Ulr05].

Atorvastatin used as a cholesterol-lowering agent for the treatment of hypercholesterolemia is a synthetic statin, 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitor, which catalyzes the rate-limiting step in cholesterol biosynthesis [Lea97]. Atorvastatin was marketed as its calcium salt (2:1) in crystalline trihydrate designated form **I** (Lipitor; Warner-Lambert, now Pfizer) [Bri97]. Lipitor became the world's best-selling medicine with sales of more than US$ 10 billion/year.

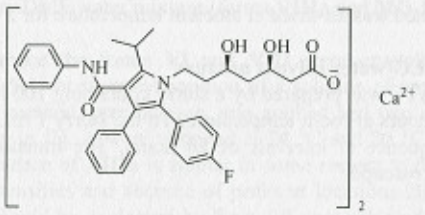

Fig. 1 Chemical structure of atorvastatin calcium (ATC).

Competitions and challenges between innovator and generic drug manufacturers prompted by the huge market size have been leading to the large number of patents concerning different crystalline forms as well as processes for amorphous forms. Surprisingly, in our literature